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16. ABSTRACT

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CHAPTER 50

Effects of Kepone on Estuarine Microorganisms

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Low concn of the insecticide Kepone, approaching those found in contaminated James River sediment, were shown to be inhibitory to the growth and oxygen uptake of microorganisms randomly selected from estuarine environments. No significant correlations were noted between growth inhibition by Kepone and cell morphology, aliphatic hydrocarbon utilization, pesticide tolerance, selected enzyme activities, nitrate reduction, and urea hydrolysis. Oxygen uptake by pure cultures grown on glucose or hydrocarbons at cell densities equivalent to 10^9–10^4 cells/ml was decreased by 60–100% at Kepone concn of 0.02–2.0 mg/liter. Total viable counts from estuarine water or sediments grown aerobically on agar media containing 0.02 mg/liter Kepone were reduced by 8–78 %. The inhibitory effect was eliminated partially when sediment populations were grown anaerobically.

INTRODUCTION

Aquatic microorganisms undoubtedly play a critical role in the metabolic transformation of organic and inorganic compounds which enter lakes, estuaries, and oceans. Some of these compounds, particularly the chlorinated hydrocarbon group, degrade very slowly in nature and consequently their fate and accumulation are of great importance in determining environmental quality. Since many chlorinated hydrocarbons accumulate in terrestial and aquatic ecosystems, they eventually could alter basic microbial degradation processes if the compounds were toxic to the microorganisms. For example, interferences in the competition among bacteria in a natural population, due to the elimination of an important member of that population, could reduce transformation efficiency and even affect the extent of transformation. The possibility that pesticide residues may have deleterious effects on microorganisms and their activities has received considerable attention (Alexander 1968; Edwards 1972; Tu and Miles 1976; Ware and Roan 1970). The emphasis of these studies, however, has been on the effects of pesticide residues on soil rather than aquatic microorganisms. Although the effects of pesticides on phytoplankton and photosynthesis has been documented (Ware and Roan 1970), information is lacking on the effects of organochlorines on aquatic microorganisms and the processes they mediate. Several important organochlorine compunds have been tested for their toxicity to aquatic microorganisms. Bourquin et al. (1975) and Bourquin and Cassidy (1975) have shown polychlorinated biphenyls to be toxic to a variety of bacteria isolated from estuarine waters. Using the agar-disc diffusion method, the investigators found that 0.1 mg of Aroclor 1242 per disc elicited definite zones of inhibition. They also showed that, by documenting physiological characteristics relative to toxicant-sensitive and toxicant-insensitive groups, certain characteristics correlated with one group more than another. Richards (pers. Comm.) has shown that pentachlorophenol in flowing sea-water systems caused the selection of PCP-tolerant microorganisms. Brown et al. (1975b) have shown that Mirex was nontoxic to aquatic

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bacteria but Kepone, a contaminant of Mirex, was toxic. Kepone may be a chemical breakdown product of Mirex (Carlson et al. 1976).

Kepone® (decachlorooctahydro-1,3,4-metheno-2H-cyclobuta (cd) pentalene-2-one), an insecticide, has been shown to be an extensive contaminant of the James River System in Virginia (U.S. EPA unpubl. data 1975). Its introduction into the James River was due to industrial effluents. Recent studies have shown that Kepone is toxic to algae (Walsh et al. 1977) and several estuarine animals (Schimmel and Wilson 1977). This paper reports on the toxicity of Kepone to estuarine bacteria.

Materials and Methods

Bacterial cultures. Bacterial isolates obtained from Range Point salt marsh (Pensacola Beach, FL), Escambia Bay surface water, and Sabine Island disposal pond were used in disc-agar sensitivity assays. These pure cultures of bacteria and fungi originally were isolated from batch culture enrichments on various carbon sources and maintained as part of our laboratory stock culture collection. Samples of water and sediment from these waters and the Gulf of Mexico were used in all mixed-culture toxicity testing. Isolates from these environments were used to determine the effects of Kepone on growth and the oxidation of specific substrates.

Reagents and chemicals. Kepone (98% pure) was obtained from Chem Services, Inc., West Chester, PA. N,N-dimethylforamide (DMF) was purchased from Aldrich Chemical Co., Milwaukee, WI. Microbiological media and substrates were obtained from Difco Laboratories, Detroit, MI.

Media. Zobell's 2216 marine medium, Z–15 (Aaronson 1970), was prepared at a final salinity of 15‰ using Rila Marine Mix (Rila Products, Teaneck, NJ). Solid medium was prepared with 2% Bacto-agar. Minimal salts basal medium (MSB) was prepared according to Stanier et al. (1966). Glucose (0.2%) was added to MSB as a growth substrate and 0.01% yeast extract (Difco) was added to compensate for any auxotrophic cultures. Kepone-Marine agar medium was prepared by adding desired concn of Kepone (in acetone carrier) to Zobell's medium after autoclaving. Acetone control plates (no Kepone) were used in all experiments.

Pure culture inhibition studies; disc sensitivity assays. Pure cultures, grown in Zobell's (Z–15) broth for two 18-h growth cycles were spread on Z–15 agar plates. Three filter paper discs (Schleicher and Schuell, 12.7 mm, No. 740-E, Keene, NH), designed for antibacterial substrate testing, were saturated with acetone or DMF solutions containing 0.01, 0.1, or 1.0 mg/liter of Kepone, dried, and then placed on the inoculated agar plate. A fourth disc contained only DMF or acetone. The actual volume of Kepone solution per disc was calculated to be 0.11 μl by determining the average increase in weight of a DMF or acetone-saturated disc.

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Mixed culture inhibition studies. Water and sediment samples collectd from James River, VA, and various estuarine and marine areas near Pensacola Beach were diluted serially in water blanks of the appropriate salinity and plated on Z–15 agar plates with and without Kepone. The plates were incubated aerobically at 28 C and colonies were counted at 4- and 8-day intervals. Sediment samples were plated similarly and incubated anaerobically in BBL Disposal Anaerobic Systems (BBL, Baltimore, MD) at 28 C for 8 days. Dominant colony types from the aerobic Kepone-containing plates were picked with sterile toothpicks and spotted onto Z–15 agar. The resulting colonies were replicated onto various differential and selective media for physiological characterization.

Oxygen uptake studies. Selected pure cultures from aerobic Kepone plates were grown in 100 ml of either Z–15 broth or MSB broth with glucose or succinate (0.2%) for 18 h prior to harvesting by centrifugation (10×*g*, 10 min). The cells were washed once with 0.05 M K_2HPO_4 buffer (pH 7.5) containing 1.5% NaCl and finally resuspended in 2 ml of saline buffer. Suspensions were kept in an ice bath until used. Oxygen uptake was determined by a Gilson Oxygraph (Model K-ICT-C, Gilson Electronics, Middleton, WI) equipped with a Clark electrode. The reaction vessel contained 1.7–1.8 ml phosphate buffer, 10–50 μl washed cell suspension (100–300 μg protein), and 10–50 mg oxidizable substrate. After a baseline level of oxygen uptake was established, 10 μl of Kepone solution (0.19–19.0 μg Kepone/μl DMF) was added to the reaction vessel and the oxygen uptake observed for any reslting effects. A positive control, 10 μl of a pentachlorophenol solution (20 mM), was then put in the same reaction vessel.

General methods. Physiological characterization of isolates on differential and selective media was carried out according to the methods of Colwell and Weibe (1970). The ability of isolates to grow on hydrocarbons (hexadecane, undecane, octadecane, benzene, naphthalene, biphenyl, xylene, toluene) was determined by placing hydrocarbon-saturated filter paper discs in the lids (for volatile hydrocarbons) or on the agar (for nonvolatile hydrocarbons) of MSB agar plates streaked with the appropriate isolate. The appearance of growth on the agar which was greater than the control plates was taken to be hydrocarbon utilization by the isolate since no other carbon source was supplied. Pesticide tolerance tests (using toxaphene, Arochlor 1242, methoxychlor, heptachlor, DDT, malathion, pentachlorophenol) were performed using the disc-agar diffusion method and recording effects on growth of isolates streaked on MSB agar containing glucose or succinate and yeast extract. Protein determinations were performed according to the methods of Lowry et al. (1951).

RESULTS

Pure Culture Toxicity Studies

The toxicity of Kepone to a variety of pure cultures from our laboratory collection was determined by the disc agar diffusion sensitivity method. Table 1 shows the degree of toxicity as expressed by the size of the zone of inhibition. Only the sensitive cultures are shown. Several of the isolates (No.'s 1, 3, 4, 11, 14, 15, 29) were particularly sensitive (as judged by the size of the zone of inhibition) to Kepone. Chemical analysis has shown Kepone to be 99% pure with no trace (less than 0.5 mg/ml) of other chlorinated compounds. Of the 26 isolates tested, 33% were inhibited at the 3.65 μg/disc concn and 50% were

TABLE 1. *Sensitivity of pure cultures to Kepone using the agar-disc diffusion method*[a]

GBERL Isolate #	Acetone Control	Concentration Kepone (μg) per Disc			
		1.46	3.65	7.3	14.6
1	0	0	+++	++++	++
3	±	+	++	+++	+++
4	0	+	+++	++++	+++
5	±	±	±	±	+
8	±	±	±	±	+
11	±	++	+++	+++	+++
14	0	0	+	++	+++
15	0	0	++	+++	+++
29	0	+	++	+++	++
36	±	±	+	+	+
42	±	+	±	++	+++
47	0	0	±	±	+
54	±	±	++	++	++

[a]0 = no zone; ± = 0–1 mm; + = 1–2 mm; ++ = 2–3 mm; +++ = 3–4 mm; ++++ = >4 mm.

inhibited at the 14.6 μg/disc concn. Fifteen percent of the isolates were inhibited at the 1.46 μg/disc concn. Higher concn of Kepone (20 μg/disc) inhibited 100% of the isolates. Of the six fungal cultures tested, one was poisoned at the 14.6 μg/disc concn. Two yeasts, *Candida maltosa and Candida lipolytica*, were both sensitive but at higher concn of Kepone than used with the bacteria tested.

Kepone-sensitive cultures (i.e., showing zones of inhibition with disc assay) showed no significant correlation ($P = 0.05$) with morphology, gram stain, hydrocarbon utilization, pesticide tolerance, amylolytic, lipolytic, and proteolytic activity, nitrate reduction, sugar utilization, and urea hydrolysis. These results are summarized in Table 2.

Mixed Culture Toxicity Studies

To determine the toxicity of Kepone to mixed populations of bacteria from a variety of marine habitats, total viable counts were performed with Zobell's seawater agar (Z–15) containing dissolved Kepone. Results of these assays (Table 3) are expressed as the percentage reduction in colony-forming units (CFU) normalized against control plates that contained no Kepone. The results demonstrate that Kepone, in concn as low as 20 μg/liter, is inhibitory to the development of colonies on an agar plate. Different degrees of inhibition (concn of Kepone which would give the same reduction in CFU's) were noted with samples taken from the same area at different times. In many cases, concn below 20 μg/liter were inhibitory. Within each population, some microorganisms were quite resistant to Kepone (i.e., grew in its presence).

Twenty cultures which grew in the presence of Kepone were selected (based on predominance) for further study. The cell types and enzymatic activities of these purified isolates showed no signigicant correlation ($P = 0.05$) with Kepone sensitivity.

Because Kepone is known to be present in sediments and detritus in concn higher than that found in the water column (U.S. EPA inpubl. data 1975), we attempted to determine the toxicity of Kepone to bacteria in sediments. The results of viable plate counts on

TABLE 2. *Physiological activities of cultures used in Kepone toxicity study*

Culture Number	Organism Type	Gram Stain	Alkane[a] Utilizer	Aromatic[a] Utilizer	Physiological Function[b]			Pesticide[a] Tolerance
					Lipo	Amylo	Proteo	
Kepone Sensitive								
1	rod	pos	+	+	−	+	+	+
3	rod	pos	−	−	−	+	+	−
4	cocc-bac	neg	−	−	+	+	+	−
5	cocc	neg	−	−	+	+	+	−
11	cocc-bac	pos	+	+	+	+	−	+
14	rod	pos	−	−	+	+	−	−
29	rod	pos	−	−	−	−	+	−
42	coccus	pos	−	−	+	−	−	−
47	cocc	neg	−	−	−	−	−	−
54	rod	pos	−	+	−	+	+	−
8	yeast	—	−	+	+	+	+	−
36	yeast	—	+	−	+	+	+	−
15	fungus	—	+	+	N.D.[c]	N.D.	N.D.	−
10	rod	neg	−	−	−	−	±	−
18	pleo	neg	+	−	+	−	−	−
20	rod	neg	+	−	N.D.	N.D.	N.D.	−
23	rod	pos	+	+	N.D.	N.D.	N.D.	+
40	cocc-bac	pos	+	−	+	−	+	+
45	rod	neg	−	−	+	−	+	−
46	rod	neg	−	−	+	−	−	+
56	cocc-bac	neg	−	−	+	+	−	+
59	rod	neg	−	−	−	−	−	−
2	yeast	−	+	−	−	−	+	−
7	yeast	−	−	−	+	+	+	−
27	fungus	−	−	+	N.D.	N.D.	N.D.	+
44	fungus	−	−	−	N.D.	N.D.	N.D.	+

[a]Hydrocarbons and pesticides listed in text.
[b]Lipo = lipolytic activity; Amylo = amylolytic activity; Proteo = proteolytic activity.
[c]N.D. = not determined.

Zobell's marine agar containing Kepone, using samples taken from Range Point marsh sediment, are shown in Table 4. Under aerobic conditions, CFU's decreased as a function of increasing Kepone concn as expected from earlier results. Under anaerobic conditions, however, the inhibitory effect of Kepone was reduced. For example, at 0.2 mg/liter of Kepone, a slight reduction (0–12%) in CFU's occurred under anaerobic conditions but a larger reduction (34–80%) occurred under anaerobic conditions. Colonies originally grown aerobically also showed decreased Kepone sensitivity under aerobic conditions. Sediment samples taken at different times generally showed different degrees of inhibition at the various concn.

The effect of Kepone on the anaerobic microbial populations in contaminated James River sediment was different. These sediments contained 0.1 ppm Kepone (A. J. Wilson, ERL-GB) when collected (8/29/77). Results of viable plate counts under aerobic and anaerobic conditions are presented in Table 4. As opposed to Range Point sediment, the

TABLE 3. *Effects of Kepone on total viable counts of bacteria from estuarine water*

Sample Source	Date[a]	Salinity (‰)	Temp (C)	CFU's × 10^2 per ml Water			
				Z-15	0.02[b]	0.20[b]	2.00[b]
Range Point	A	15	21	181	108(41)[c]	73(60)	35(81)
	B	15	24	86	65(25)	35(60)	33(61)
	C	20	23	1	12(28)	13(23)	13(25)
Escambia Bay	A	11	22	267	81(70)	233(13)	130(52)
	B	15	24	13	7(50)	10(26)	5(66)
	C	18	22	12	11(8)	9(23)	9(23)
Laboratory Pond	A	20	20	479	448(7)	409(14)	396(18)
	B	15	26	104	49(53)	–	19(82)
	C	20	27	544	501(8)	437(20)	309(43)
Gulf of Mexico	A	33	18	270	62(78)	32(89)	46(—3%(
	B	33	22	13	10(21)	10(25)	8(40)
	D	33	24	15	6(59)	1(92)	1(95)

[a]Sampling Dates. A = 4/10/77; B = 4/17/77; C = 4/29/77; D = 5/7/77.
[b]Mg Kepone/liter used in Zobell's marine agar.
[c]Numbers in parentheses are percentage reduction in CFU's normalized against growth on control plates of Zobell's marine agar containing no Kepone.

TABLE 4. *Typical effects of Kepone on the production of colony-forming units from Range Point marsh and James River sediments*

Sediment Source	Incubation Conditions	CFU's × 10^2/g Sediment[b]			
		Control Z-15	Kepone (mg/l) 0.02	0.2	2.0
Range Point	anaerobic	34	41(0)[c]	41(0)	37(0)
	aerobic	210	200(5)	140(34)	30(86)
James[a] River	anaerobic	43	28(35)	24(44)	9(79)
	aerobic	15200	16500(0)	14200(7)	9400(36)

[a]James River sediment contained 0.1 ppm Kepone.
[b]CFU's are the mean of the results of triplicate platings. Experimental error in these assays averaged ± 8 CFU's.
[c]Numbers in parentheses are percent reduction in CFU's relative to Z-15 control.

James River sediments contained bacteria which did not show decreased sensitivity to Kepone under anaerobic conditions.

Oxygen Uptake Studies

To assess further the sensitivity of bacteria to Kepone, we conducted oxygen uptake studies. As seen in Table 5, oxygen uptake was a sensitive indicator of Kepone toxicity. The isolates showed varying degrees of sensitivity: most cell suspensions (10^6–10^7 cells/ml)

TABLE 5. *Effect of Kepone on oxygen uptake by resting cell suspensions of isolates from estuarine environments*

Isolate No.	Source[c]	Kepone Enrichment Concentration	Percentage Reduction in O_2 Uptake[a] Kepone (mg/l)[b] 2	20	200
49	EB	0.00	+25	100	100
51	EB	0.02	0	100	100
61	EB	0.20	0	100	100
56	EB	2.00	66	75	66
32	RP	0.00	0	83	31
35	RP	0.02	11	100	100
43	RP	2.00	—	64	33

[a]nM O_2/ml/min/μg protein or glucose.
[b]Kepone concn required to show inhibition of concd cell suspension (10^6–10^7 cells/ml). One thousandth of this concn would inhibit O_2 uptake by bacterial populations in Escambia Bay (10^3–10^4 cells/ml).
[c]EB = Escambia Bay, RP = Range Point.

were inhibited at the 20 mg/liter concn, whereas relatively little inhibition occurred at the 2 mg/liter concn. Inhibition at 20 mg/liter, in many cases, was substantially greater than at the 200 mg/liter (isolates 32, 43, and 56). It also should be noted that some isolates actually increased oxygen uptake in the presence of Kepone (isolate 49).

Discussion

Our results indicate that Kepone is toxic to microorganisms. Kepone concn as low as 0.02 mg/liter reduced the number of colony-forming units on Zobell's marine agar by 23–92 % . This is ten to a hundred times more toxic than the PCB's (Bourquin 1975), chlordane (Trudgill et al. 1971) and heptachlor (Shamiyeh and Johnson 1973). Kepone levels in James River sediment have been shown to be 0.05–0.5 mg/kg, particularly in areas of high organic content (U.S. EPA unpubl. data 1975). Since Kepone is resistant to chemical and biological degradation (Garnas, Bourquin and Pritchard, unpubl. data) and since Kepone appears to be washing out of the James River system at a very slow rate, residual Kepone could have an inhibitory effect on bacteria in the river.

However, this compound was not universally toxic to all cultures at the concn tested. Since only certain members of the bacterial populations were inhibited by Kepone at concn levels equal to those in the James River, it is important to know if the tolerant species will replace the sensitive species and thus maintain the metabolic integrity of the ecosystem. In general, this replacement process occurs in both soil (Tu and Miles 1976) and aquatic (Ware and Roan 1970) environments. Unpublished results from our laboratory, however, have shown that Kepone, at low concn, decreases the rate at which the pesticide methyl parathion is degraded, thus implicating a possible interference with the metabolic integrity of an aquatic system.

Of the three methods used to determine toxicity in this study (i.e., sensitivity disc, plate counts, and oxygen uptake), each appear to yield similar toxicity results. Plate counts are the easiest method to assess toxic effects on a mixed population of bacteria, and the minimum inhibitory concn (MIC = 0.02 mg/liter) gives a good reflection of the effects

possibly occurring under natural conditions. Little can be said about the MIC from agar-disc diffusion experiments except that low concn of Kepone are inhibitory.

Much higher concn of Kepone would appear to be necessary to inhibit oxygen uptake. However, these studies employed high densities of cells (10^6–10^7 cells/ml). These levels are, in fact, proportional to an environmental level. Escambia Bay waters have a cell density of 10^3–10^4 cells/ml. We have determined, in plate assays, that the growth of this population is inhibited at 20 μg/liter Kepone. By concentrating both factors a thousand times, 10^6–10^7 cells require 200 mg Kepone/liter to effect inhibition, which are the same levels employed in oxygen uptake studies.

Physiological characterisitcs (sugar fermentation, enzyme activities, urea hydrolysis, hydrocarbon oxidation, etc.) as possible tools to determine the toxic effects on specific physiological processes have not been used extensively (Brown et al. 1975a). Our study demonstrates no significant correlation between these characterisitcs and Kepone toxicity. Amylolytic activity was prevalent in some Kepone-sensitive organisms as was the case for PCB-sensitive bacteria (Bourquin and Cassify 1975). These effects apparently cannot promote the enrichment of amylase-positive organisms in a mixed population because neither static nor flow-through systems exposed to Kepone in our laboratory showed this type of enrichment.

The nontoxic effect of Kepone on bacteria grown anaerobically points to a possible involvement with electron transport and respiration. Widdus et al. (1971) and Trudgill et al. (1971) have shown that the inhibition of NADH oxidase activity in *Bacillus subtilis* by chlordane occurred indirectly through the apparent disruption of membrane-mediated electron transport. Similar mechanisms could account for the effect of Kepone in anaerobic versus aerobic conditions. Trudgill et al. (1971) did not study the effect of chlordane on anaerobic growth, but he showed that a *Streptococcus* species (which would have minimal cytochrome-mediated electron transport activity) was unaffected by chlordane. Kepone, therefore, could interfere with membrane-mediated transport.

A possible explanation for the increased oxygen consumption in the presence of Kepone is an oxygen uncoupling effect which would generate hydrogen peroxide. Hydrogen peroxide would then be split by catalase, releasing O_2 gas. The mechanism accounting for the release of oxygen by cells is still under investigation.

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